THE EFFECTS OF DIETARY SELENOMETHIONINE ON THE ESCAPE BEHAVIOURS OF FATHEAD MINNOWS

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Philip James Anderson

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Abstract

Selenium is both an essential nutrient and a toxicant for animals, with only a relatively small concentration change separating the two. Toxicological work has reported various effects of selenium on fishes, including developmental impacts and deformities of the musculature and sensory systems. Behavioural ecotoxicology, a more sensitive study of toxicity, has also provided evidence that sublethal concentrations of selenium are having measurable impacts, such as negatively affecting swimming behaviours, in real-world ecosystems.

In this thesis, I assessed the impacts of a selenomethionine-laden diet on the escape behaviours of the Fathead Minnow. Using kinematic analysis, I observed how fish responded to various looming threats. I exposed fish to sub-chronic periods of environmentally relevant concentrations of selenium in the form of selenomethionine-spiked diets. I achieved whole-body concentrations that approach Canadian tissue-specific guidelines for wild fish populations. In my first experiment, I used a weight drop to test the fish's ability to respond to a mechanosenory stimulus and the performance of their fast-start response. My second experiment focused on the impacts of selenomethionine on visual acuity and how it affects visual perception of a threat. I also investigated how exposed fish would recover from any potential impacts when returned to a contaminant-free diet.

My results indicated there was no significant effect of selenomethionine on either the visual response to a threat, or burst swimming behaviours of the fast-start response in Fathead Minnows. Additionally, there were no latent changes to Fathead Minnow escape behaviour throughout the recovery period. These results were contrary to both my predictions and the literature that showed critical swimming behaviour was compromised in selenomethionine - exposed freshwater fish. My work helps to show that the effects of toxicants on behaviours can be highly specific and cannot be generalized.

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Chapter 1: Introduction

Selenium was first discovered in 1817 by the Swedish chemist Jacob Berzelius, who thought it was a toxic compound. However, in the 1950's, scientists realized that selenium is both an essential nutrient and a toxicant for animals. Schwarz and Foltz (1957) were the first to show that selenium could protect against liver necrosis. In the 1970's, selenium was shown to have antioxidant properties as an essential component of glutathione peroxidase, an enzyme that reduces free radicals to protect the organism from oxidative stress (Rotruck et al. 1973). However, when selenium is found in excess in organisms, it can have harmful effects through carcinogenesis, cytotoxicity and genotoxicity (Sun et al. 2014). Herein lies the fickle nature of selenium: at concentrations too high or too low it can harm the individual, but when the correct dose is achieved it can protect the cells from damaging oxidation.

Selenium naturally occurs in soils and ores, releasing the trace amounts required for life via normal weathering cycles. Such low levels of selenium can support biotic communities and provide them with antioxidant protection. However, human development has drastically increased the amount of selenium available to natural systems via both controlled and uncontrolled releases from a number of industries including coal, petroleum, uranium, and agriculture (Frankenberger and Engberg 1998). Once this additional selenium has been released into a system, toxic effects can be observed decades later (Lemly 1997).

Selenium is a contaminant of concern because of its ability to rapidly bioaccumulate from low concentrations in the water or soil to high concentrations in animal tissues and can lead to the total collapse of populations (Hamilton 2004). Chronic exposures have the potential to collapse populations even without outwardly visible signs such as mass mortality events. For instance, selenium primarily affects the reproductive functioning of aquatic vertebrates. Oviparous species pass selenium from mother to developing young through their eggs and this leads to deformities in juvenile animals, with the potential to cause total recruitment failure (Lemly 2002). Exposure to sublethal concentrations of selenium through water or food uptake can also impact normal development and performance of aquatic vertebrates such as fish, amphibians, and birds (Bennett et al. 1986, McPhee and Janz 2014).

Predation is among the strongest evolutionary driving forces in the natural world. It can exert its pressure on a variety of levels. On a large scale, introduced predators have the capacity

to extirpate native populations, changing the community dynamics of a system (Nicholson et al. 2015). It can also act on individuals, altering body morphology to reduce the threat of gape-limited predators (Bronmark and Miner 1992). Even the chemical traces of predators can have profound effects on the behaviour of animals (Ferrari et al. 2010). The development of behaviours to protect individuals from being consumed occurs in nearly all species of animals (Elgar 1989, Lima and Dill 1990, Ferrari et al. 2010).

Fish have a wide array of predator avoidance strategies and behaviours. For instance, they alter when and where they forage and reproduce in response to risk (Ferrari et al. 2010). They may also change their body shape, develop cryptic colouration or develop protective armour and spines (Keenleyside 1979, Abrahams 1995, Chivers and Smith 1998). However, once a predation event is imminent, they have few options other than to respond with a rapid movement or change of direction (Domenici and Blake 1997). These routine swimming behaviours can be extremely sensitive to anthropogenic change, and often deviate from normal at much lower concentrations than what is observed for traditional toxicology endpoints (Little and Finger 1990). The behavioural ecotoxicology of selenium and swimming behaviour can provide insight into how sublethal levels of selenium are altering the ecological functioning of fish when they are exposed to environmentally relevant levels of contamination.

1.1 Selenomethionine Toxicity

Selenium is a non-metal chemical element that naturally occurs in soils and ores. Selenium deposits are located around the world and selenium is naturally released into aquatic environments in trace concentrations by weathering. These levels are often enough to sustain local animal populations, which only need small amounts of selenium to meet their dietary requirements. Industrial and commercial applications of selenium include the production of solar panels, photovoltaic devices, nutritional supplements, and additives in alloys, dyes, and inks. Selenium contamination is also a by-product of many extractive resource industries on which our reliance is increasing.

Biological function

Selenium is not an essential nutrient for all life. Some simple organisms, such as yeast, have no proteins or pathways capable of incorporating selenium into their cells, but it has been

shown to be a vital requirement for all animals (Flohé et al. 2000). Selenium is utilized by various body systems such as the immune, endocrine, reproductive, and neurological systems (McKenzie et al. 1998, Köhrle 1999, Flohé et al. 2000, Colangelo et al. 2014). Selenium is predominantly incorporated into amino acids, and has a key role in the synthesis and folding of selenoproteins. Selenoproteins have a wide variety of uses, but are primarily used to protect the body from free radicals, which have the potential to cause irreversible harm to the organism (Zarczynska et al. 2013). Selenium, along with vitamin E, prevents dystrophy of muscle tissue, by eliminating the hydrogen peroxide and hydroperoxides that cause oxidative damage to cells and membranes (Watanabe et al. 1997). Fish generally require between 0.1-1.2 mg/kg dry weight (DW) in their diet to meet their nutrition requirements (Antony Jesu Prabhu et al. 2016). Excessive levels of selenium, most often accumulated through the diet, can lead to impaired respiration, circulation, sensory function, excretion, reproduction, and teratogenic deformities of fish due to increased oxidative stress that damages cells (Lemly 2002, Janz et al. 2010).

Sources of selenium

Selenium can be released into an aquatic system from both natural and anthropogenic sources. The majority of selenium contaminations are linked to mineral mining and processing (Lemly 2004). Copper, uranium, and coal can all contribute significant quantities of selenium into water systems. Selenium is bound within these minerals, and extracting them allows for the selenium to leach out when water percolates through the ores (Lemly 2004). Acute and chronic selenium exposures can also occur from metal smelting operations, landfills without proper waste management, petroleum refineries, and irrigation of seleniferous soils (Lemly 2004).

Selenium occurs in two forms in nature, inorganic and organic. Inorganic forms, like selenite and selenate, are the most prominent species in the effluents from industrial development (Janz et al. 2014). Selenite is more toxic to aquatic invertebrates and fish, and accumulates more rapidly and in higher quantities than selenate (Hamilton 2004). Selenite dominated waterbodies are at greater risk of experiencing selenium bioaccumulation than selenate dominated waters (Hamilton 2004). Organic forms, like selenocysteine and selenomethionine, are the most common species in the diet of aquatic organisms (Fan et al. 2002). Bioavailability varies among species of selenium. Supplementation with organic forms of selenium, mimics the uptake of selenium from natural prey items more closely than inorganic selenium (Hamilton 2004).

Selenomethionine is the most biologically available form of selenium, partly the reason for its prevalence in selenium toxicology studies (Besser et al. 1993). By utilizing selenomethionine laden diets instead of inorganic exposed algae or zooplankton, research on how selenium absorbed through the diet can be simplified.

The United States Environmental Protection Agency (U.S. EPA) and the Canadian Council of Ministers of the Environment (CCME) both understand that anthropogenic selenium is a significant risk to human and ecosystem health, and they have both implemented limits on the release of selenium in industrial effluent. The U.S. EPA uses a system that sets limits on the levels of selenium found in the water (1.5 µg/L for lentic and 3.1 µg/L for lotic waters) and in whole body samples of fish (8.5 mg/kg DW) (U.S. EPA 2014). Regulation differences for still and flowing water comes down to the increased feeding and potential for bioaccumulation fish experience in larger bodies of water relative to smaller streams that are generally used for spawning. Selenium is provincially regulated in Canada, where most provinces refer to the CCME recommendations for water at 1 µg/L, but British Columbia has developed their own guidelines similar to the U.S. EPA (2 µg/L water and 4 µg/g DW body tissue) (Health Canada and Environment Canada 2015). There is much debate over what type of regulations should be used for monitoring the impacts of selenium on freshwater systems (DeForest et al. 1999, Hamilton 2002). These limits are designed to regulate industries to release the lowest amount of selenium technically and economically possible to protect the environment and biodiversity (Health Canada and Environment Canada 2015). Changes in behaviour can be elicited by exposure periods 75% shorter and 98% less concentrated than those that would induce mortality in fish in other contaminants making behavioural endpoints an important consideration in contaminant regulation (Little et al. 1990, Kane et al. 2005).

Selenomethionine toxicity in fish populations

When selenium is absorbed by some organisms, it is not able to be metabolised and can be deposited within their tissues, leading to bioaccumulation (Zhang and Gladyshev 2009). Bioaccumulation can occur through two mechanisms: bioconcentration and biomagnification. Bioconcentration is the uptake of water-borne substances into the organism through the skin or tissues. Biomagnification is the increase of the concentration of a substance in the tissue of organisms up trophic levels. It has been shown that the majority of selenium bioaccumulation

occurs through the diet rather than water-borne selenium uptake in higher trophic levels (Hamilton 2004). Bioconcentration generally has the greatest effect on small invertebrates and plants, where their surface area relative to their body size is larger. Freshwater invertebrates and plants are more tolerant of selenium, allowing them to accumulate large quantities and make it available for higher trophic organisms (Stewart et al. 2010). Invertebrates and plants uptake the inorganic selenium from the water and convert it to organic forms of selenium such as selenomethionine, the most biologically available form for fish (Phibbs et al. 2011).

By consuming plants and invertebrates with high levels of selenium, higher trophic organisms will biomagnify selenium. Excessive selenium in the tissues of fish and other animals can be extremely toxic, and leave the individuals or their offspring with deformities and reduced fitness (Lemly 2002). Selenium can therefore cause population declines and extirpate species through reproductive and recruitment failure. Sublethal selenium exposure can produce deformities like curved spinal columns, muscle atrophy, cataracts, and exophthalmos, which is swelling of the soft tissue in the skull and distends the eyes outwards (Lemly 2002). In chronic laboratory exposures, selenium has impaired a wide variety of biological functions in fish, such as swimming performance, energy metabolism, and visual perception (Thomas and Janz 2011, McPhee and Janz 2014, Raine et al. 2016). Larval fish have been shown to accumulate selenium in the developing eye tissues, suggesting that they are at risk of decreased visual performance when selenium levels are elevated (Choudhury et al. 2015).

Noteworthy examples of selenium contamination

Belews Lake, North Carolina received effluent and coal fly ash leachate from a coal-fired power plant for over a decade before the power plant began to manage its selenium contamination (Lemly 2002). However, it was not until 1976 when no young-of-the-year for most fish species were located that the alarm was raised (Harrell et al. 1978). The fish community had shifted to more selenium-tolerant species, including Fathead Minnows (*Pimephales promelas*), Black Bullheads (*Ictalurus melas),* and Green Sunfish (*Lepomis cyanellus*) (Hamilton 2004). Subsequent studies of this system focused on the symptoms of selenium exposure, biogeochemical cycling of selenium, and the long-term persistence of selenium (Lemly 1997, 2002). The results generally indicated that the rapid bioaccumulation of selenium into fish populations and few outwardly visible signs of community change were the

root causes of the collapse, and that selenium should be taken as a serious issue with freshwater resources (Lemly 2002).

Development and industrialization of land resources, be it for mining or agriculture, is a common vector for the mobilization of contaminants into water bodies (Lemly 2004). Increased irrigation of seleniferous soils over drainage tiles is the leading source of selenium into the groundwater of the San Joaquin Valley of Central California (Fio et al. 1991). Irrigation runoff flows into groundwater, which proceeds to empty into the various wetlands and pond systems in the region, a major wintering area for waterfowl (Presser and Ohlendorf 1987). In the mid-1980's, selenium concentrations in waterfowl eggs showed that the parents were facing significant bioaccumulation and led to the widespread failure of embryos and hatchlings, with up to 60% of all nests displaying mortalities in some species (Presser and Ohlendorf 1987). The control and management of the wastewater, primarily through dilution and evaporation methods, has led to drastic declines in selenium concentrations in water and biota (Kausch and Pallud 2013).

Key Lake, Saskatchewan, and the surrounding area has been the focus of extensive monitoring and experiments on the effects of selenium, among other contaminants, in the discharge from a large-scale uranium processing and milling facility (Janz et al. 2014). The levels of selenium in the water, sediment and biota of lakes and rivers in the region have been assessed by Muscatello and Janz (2009) working in conjunction with the corporation that operates the mine. By using an ecosystem-level perspective on the issue, there is now a better understanding of how selenium travels from a point source into an aquatic system. It is now possible to carry out rigorous in situ experiments to better understand the fate of selenium in organisms and the environment (Goertzen et al. 2011, Phibbs et al. 2011, Janz et al. 2014). Long-term monitoring of landscapes receiving contaminated effluent can help to establish guidelines on how selenium changes as it travels through an ecosystem (Janz et al. 2014).

1.2 Escape Behaviours

Predation and the threat of it can shape animal behaviours in drastic ways. Both the actual act of physical consumption and the stress resulting from the threat of predation can greatly influence behaviour (Lima and Dill 1990, Ferrari et al. 2010). Signalling predation threats in an aquatic world where visibility may be limited can be difficult, but the use of chemosensory

signals and cues can help to protect conspecifics in these conditions (Chivers and Smith 1998). Chemical cues in the skin cells fish are released when the skin is damaged during predation and can trigger a number of distinct behaviours from freezing and shelter use in prey species to increased foraging and prey searching in predators who exploit the chemical signal of an injured prey fish (Chivers et al. 1996, Chivers and Smith 1998). These chemical compounds are known as alarm cues. When prey fish sense predation events occurring in close proximity through the chemoreception of alarm cues, they enter a state of high alert. They stop foraging and seek the protection of a shelter to reduce their likelihood of falling victim to a predator (Ferrari et al. 2010). The trade-off between routine behaviour and antipredator behaviour is incredibly important to fish (Lima 1998). A highly efficient and precise response to predators is critical to ensure that the fish wastes the least amount of energy in the response and returns to routine foraging as soon as possible. Wasting energy in a predator response can have cascading issues for the growth and functioning of the fish.

Antipredator behaviours consist of a broad spectrum of responses to the threat of consumptive harm. These behaviours can either be innate in the fish (Ferrari et al. 2010) or their development occurs as soon as the fish becomes free-swimming, a stage of rapid growth and high vulnerability to predators (Fuiman and Magurran 1994). Escape behaviours are of critical importance to prey fish because they are life and death scenarios. The fast-start response of teleost fish allows them to rapidly change direction and displace themselves from their original location.

Fish locomotion

The study of fish swimming behaviour has many applications to biology and beyond, from anthropogenic change to robotics to xenophobia (Landa 1998, Liu and Hu 2010, Allan et al. 2013). There are three broad categories of fish swimming types, classified by their duration: sustained, which lasts greater than 200 minutes; prolonged, 20 seconds to 200 minutes; and burst, less than 20 seconds. Fish have two types of muscle in their bodies. Red muscle or slow-twitch fibres are responsible for the slow and steady swimming that the fish routinely uses. This type of muscle primarily uses aerobic respiration to produce the energy needed to contract the fibres and power the fish. White muscle, or fast-twitch fibres, primarily uses anaerobic respiration to power its contractions, and is used when fast and intense muscle responses are

required (Goolish 1991). Sustained swimming behaviours primarily rely on the contractions of the red muscle, or slow oxidative fibres, in the fish's body and does not end in fatigue (Jayne and Lauder 1994). The prolonged swimming behaviour is faster than the sustained swimming behaviour, dependent on the contraction of red muscle, or fast oxidative-glycolytic muscle, but ultimately ends in fatigue. The final category, burst swimming, utilizes both the red and white muscle fibres, or fast glycolytic fibres, but can last only for a short duration before fatigue sets in (Jayne and Lauder 1994).

The Mauthner cell system and the C-start response

The escape response of the teleost fish is dictated by a series of large-bodied neurons, called the Mauthner cells. Their large size allowed for early study of their function in neurology and neuroethology (Diamond 1971). The C-start and the firing of the Mauthner cells have been closely linked, such that they can be used to study the connection between neural pathways and behavioural patterns (Eaton et al. 2001). The system begins with a stimulus to the fish, both vibrations and visual stimuli have been shown to produce a response (Zottoli 1977, Fuiman and Cowan 2003). This stimulus excites the neurons and the signal travels through the axon and into the spinal cord. The cells in the Mauthner cell system are responsible for converting the perceived stimulus into a muscle response.

The C-start has three phases, as defined originally by Weihs (1973). It was first described as an "L"-shaped response, but the convention in the field has become to identify these fast-start behaviours as C-starts (Domenici and Blake 1997). The first stage is the preparatory stroke, where the Mauthner cell response produces a contraction in the body wall opposite the stimulus creating a "C" shape with the fish's body. The second phase is the propulsive stroke, which generates the thrust needed for the direction change and displacement. The contralateral body wall contracts to straighten the tail out, thrusting the fish away from the initial position. The final phase is when the fish regains a normal cadence of swimming. The behaviours in this phase are variable, the fish can display a prolonged swimming pattern, another rapid turn or gliding along the escape trajectory (Weihs 1973). There are a number of definitions of the phases of the C-start in the literature. Distinctions between phases can be made based on the direction of travel, the onset of propulsion, and electromyography signals (Kasapi et al. 1979, Foreman and Eaton 1993, Jayne and Lauder 1993).

Impacts of anthropogenic change

Human-linked stressors have been shown to impact a broad array of swimming behaviours in fish (Rajotte and Couture 2002, Allan et al. 2013, McPhee and Janz 2014, Simpson et al. 2015b). Swimming capacity and activity are two of the most commonly studied behavioural endpoints. Swimming capacity is the measure of the fish's ability to swim against a current, often described with critical swimming speeds (Little and Finger 1990). Swimming activity describes altered swimming behaviours such as movement, turning, and position (Little and Finger 1990). Stressors that hinder normal development, such as metal contamination, or those that influence physiological functioning, such as water temperature or dissolved CO_2, can impact both the swimming activity and capacity of fish (Goertzen et al. 2011, Allan et al. 2017).

Anthropogenic climate change is leading to changes in the oceanic environment, increasing temperatures and CO_2 concentrations in the water. These changes are having significant effects on the physiology and behaviour of ocean-dwelling fish (Munday et al. 2012). Recent research has investigated the impacts of temperature and CO_2 increases on the kinematic response of coral reef fish (Allan et al. 2013, 2017). CO_2-exposed prey fish were shown to significantly decrease the distance travelled during their escape and also decrease their reaction distance (Allan et al. 2013). They hypothesized that this change was due to the effect of CO_2 on the motivational component of the escape response, suggesting that the sensory-motor performance and timing of the Mauthner cell reaction was altered (Allan et al. 2013). This study was followed up by an assessment of the impacts of both increased temperature and CO_2 on a similar species of coral reef fish. Warming had a greater effect on the fish's escape ability than CO_2 (Allan et al. 2017). They postulated that the temperature stress altered the muscle power of the fish and led to the observed changes in escape performance. Here we see that two stressors that affect different physiological systems can both lead to a depressed escape response. Changes to any one of the interconnected systems within the escape response can lead to a breakdown of the response as a whole.

1.3 Study Species: *Pimephales promelas*

Fathead Minnows are a common study species in toxicology and ethology, and are available in Saskatchewan throughout the open water season. They inhabit a wide range of freshwater aquatic systems, ranging from small ponds to large, slow-moving rivers. They are

generally tolerant of a variety of water chemistries and characteristics (Scott and Crossman 1973) and readily tolerate life in aquatics facilities. Fathead Minnows are a common prey species for a number of piscivorous fish, including Northern Pike (*Esox lucius*), Lake Trout (*Salvelinus namaycush*) and Walleye (*Sander vitreus*), as their soft ray-finned body is easy to consume (Wahl and Stein 1988). They are an important link between the producers and the predators in aquatic systems. The sport fish industries they support can generate billions of dollars a year for Canada and the United States, but they are also a valuable commodity in the baitfish industry (Litvak et al. 1993, Fisheries and Oceans Canada 2012)

Minnows can uptake selenium from their diet and the environment, which leads to physiological and developmental defects (Pyron and Beitinger 1989, McPhee and Janz 2014). Small omnivorous fish, like the Fathead Minnow, play an important role in the trophic transfer and bioaccumulation of selenium. They consume the lower trophic organisms, such as algae and microorganisms, who uptake and transform waterborne selenite and selenate into the more biologically available organoselenium compounds (Stewart et al. 2010).

1.4 Research Objectives

In this thesis, I will investigate how selenomethionine affects the predator escape behaviours of the Fathead Minnow. My work will investigate the kinematics of the fast-start response and the ability to respond to an impending threat when Fathead Minnows are exposed to selenomethionine-spiked diets. I will present two data chapters, summarizing two distinct experiments that assess the impacts of selenomethionine on the motor and sensory systems associated with the escape behaviours of the Fathead Minnow. By observing how contaminants impact communities in ways other than mortality, we can better understand how systems handle stressors and become more resilient to disturbances in the future. Using behavioural ecotoxicology as a tool to assess the impacts of regulated contaminants on aquatic systems, it provides us with finer scale information about how the communities are functioning relative to traditional toxicology endpoints.

How does selenomethionine impact the kinematics of an escape response? In my first experiment, I assessed the impact of a selenomethionine-enriched diet on the escape behaviour and performance of the Fathead Minnow. In part A, I looked at how different concentrations of selenomethionine in the diet affected the performance. This portion of the experiment allowed

me to refine my protocols and technique. Fish in bodies of water receiving low concentrations of selenium may not be experiencing obvious toxicity effects, such as mortality or deformities, but their swimming may still be affected. In part B, I was also interested in comparing how the exposure period altered the impacts of escape behaviour. I had three diets with different concentrations of selenomethionine and two different exposure periods. In addition to these treatments, I wanted to determine if the fish had the potential to recover from our exposure periods. Potadromous fish, those that complete migration cycles within freshwater, will often move into headwater streams, where an effluent would have a much greater influence on the selenium concentration of the ecosystem. Studies have shown that fish are able to depurate selenium when the exposure ends, for example when a fish migrates out of an area currently or previously receiving selenium effluent (Bertram and Brooks 1986, Besser et al. 1993). Their return to larger bodies of water, with lower concentrations of selenium, will likely result in a lower body burden, but the recovery of performance is unknown.

How does selenomethionine impact the response to visual threat? In my second experiment, I conducted trials to determine the effect of a selenomethionine-enriched diet on the ability of Fathead Minnows to respond to a looming visual threat. We used diets spiked with three different concentrations of selenomethionine and two different exposure periods. Physical deformities of the eyes have been observed in selenomethionine-exposed wild populations and visual processing has been altered in lab experiments, therefore there may be a decrease in the ability of Fathead Minnows to detect visual threats (Lemly 2002, Raine et al. 2016). As in experiment one - part B, we also assessed the potential for the fish to recover from their exposure. Fish have the potential to repair visual sensory tissues that have been damaged due to physical injury (Zupanc 2009), but the repair of chemical damage is not as well understood.

1.5 Ethical Statement

The following studies were approved by the University of Saskatchewan's University Committee on Animal Care and Supply (protocol # 20160045). Wild fish were collected under a Saskatchewan Ministry of Environment Special Collection Permit issued to Dr. Doug Chivers. All fish were humanely euthanized with an overdose of tricaine methanesulfonate (MS-222) followed by a blow to the head after their use in experiments.

Chapter 2: The effects of dietary selenomethionine on the fast-start response of the Fathead Minnow

2.1 Introduction

In our everchanging world, aquatic systems are receiving significant stressors from anthropogenic development. Like other trace elements, selenium is both an essential nutrient and a toxicant to animals, with only a small change in concentration differentiating the two. Extractive resource industries are releasing small amounts of selenium to surrounding water bodies, which are bioaccumulating up the trophic system (Lemly 2004). Fish in selenium-contaminated systems are facing threats to their population structure but also to their behaviour and physiology.

Predation is an extreme driving force in community composition and can shape individual behaviour, morphology, and physiology (Ferrari et al. 2010). Fish have developed a wide array of antipredator behaviours to reduce the likelihood of a predator attack. However, once a predator strike is impending, an escape response is the only option. Teleost fish have a well-developed reaction to looming stimuli, called a C-start response (Domenici and Blake 1997). This response allows for rapid directional change and displacement to avoid an approaching predator. The C-start links the sensory, neural and locomotive systems of the fish to produce a reliable response (Domenici and Blake 1997). Hair cells along the lateral line of the fish detect the vibrations from an approaching stimulus (Eaton et al. 2001). This triggers the Mauthner cell system to fire. The Mauthner cell system is responsible for eliciting the fast-start response, it coordinates the muscle response on the contralateral side of the body to move the fish out of the way of the threat (Eaton and Emberley 1991).

Toxicants can affect swimming performance at exposures as low as one third of the exposure required for mortality (Little and Finger 1990). Therefore, it is tremendously important to study how levels of toxicants less than the typical LC50 and LD50 affect the behaviour and ecological functioning of fish. Selenium has been shown to impact the sustained swimming capacity of freshwater fish by decreasing their critical swimming speed, tail beat amplitude and tail beat frequency at ecologically relevant concentrations, 3.7 -9.9 µg/g Se dw for 60-90 days (Thomas and Janz 2011, McPhee and Janz 2014). Critical swimming performance is a measure

of a fish's ability to swim at a steadily increasing pace up until the point of fatigue (Brett 1964). A decrease in muscle function is the hypothesized cause for the decrease in swimming capacity. Selenium substitution in disulfide bonds altering protein synthesis and oxidative damage are presented as the mechanism for the decrease (Thomas and Janz 2011). There are also significant impacts to metabolic functioning and energy storage, suggesting that the aerobic functioning is compromised in selenium-exposed fish (McPhee and Janz 2014). Impacts to swimming capacity have been linked to increased predation in freshwater fish (Weis and Weis 1995, Painter et al. 2009). Studies of other stressors, such as CO_2 and warming, have been shown to specifically impact the burst speed swimming performance of the escape response (Allan et al. 2013, 2017).

In this experiment, I explored the effects of dietary selenomethionine on the fast-start response of Fathead Minnows. Fish were fed a selenomethionine-spiked diet for various sub-chronic exposure periods. They were tested for their ability to perform an escape response in specially designed burst escape chambers. I measured each fish's latency to respond to the stimulus, velocity, acceleration, distance travelled, angle of escape, and angle of body bend during the response to observe how different selenium concentrations impacted the escape response. I also investigated the ability of the fish to recover from the selenomethionine diet when a clean diet was reintroduced. I hypothesized there will be a significant effect of selenomethionine on the escape response. I predict that I will observe a decreased kinematic response and an increased latency to respond to the threat, because of the reported impacts to the muscle function and metabolism (McPhee and Janz 2014). I also hypothesized that the fish will display a significant change to the escape response over the recovery period. My prediction is that as the selenium is depurated by the minnow (Bertram and Brooks 1986), the fish will recover from the stressor and display a typical response to the threat. Recovery of other behaviours have been previously observed in contaminant exposed fish (Baker and Montgomery 2001).

2.2 Methods

Study Species Collection and Care

Fathead Minnows were collected using seine nets in the summer of 2016 from Pike Lake, SK. Pike Lake is an oxbow lake of the South Saskatchewan River with a diverse predator community including aerial, aquatic and terrestrial invertebrate and vertebrate predators. Wild

fish from Pike Lake were selected for this study to ensure that individuals had experience with a wide array of predation strategies, and would display an escape response when presented with an uncertain threat. Despite Fathead Minnows being a species tolerant of selenium (Hamilton 2004), their common use in toxicology and ethology allowed for my results to be comparable to other work in the field.

Following capture, the minnows were transported to the R.J.F. Smith Centre for Aquatic Ecology and housed in a 1700L tank filled with dechlorinated municipal tap water. Fish were kept on a 16:8 h light:dark cycle, and fed commercial fish flakes daily. Approximately two weeks prior to the exposure period, fish were randomly transferred to 2.5 L aerated tanks, in size-matched pairs and maintained at approximately 20° C, on a 16:8 h light:dark cycle. Fish were fed finely ground commercial fish flakes daily to mimic the consistency of the experimental diets. 60% water changes were run daily by a programmable pump. Fish were kept at temperatures below spawning conditions, and no obvious secondary sexual characteristics (fat pads and nuptial tubercles) were observed during the duration of the experiment. The experiment was carried out over the course of two years, part A occurred during the summer and fall of 2016 and part B occurred during the summer of 2017. Part A was run in two sections; the first was from August 2nd, 2016 until September 9th, 2016 and the second was from October 25th, 2016 until December 2nd, 2016. Part B was run in a single section, starting on May 15th, 2017 and ending on September 13th, 2017. Staggered starts were used to ensure that each fish could be run at the end of the exposure period. This allowed me to run each fish at the precise end of its exposure period to maintain consistency across the length of the experiments.

Diet Preparation and Exposure

Selenomethionine-spiked diets were prepared by combining commercial fish food (Nutrafin Brand Max Goldfish flakes) with different concentrations of selenomethionine (Purchased from Acros Organics). In part A, I used three diets with nominal concentrations of 0 (control), 6 (low) and 12 (high) µg Se/g food. In part B, I used three diets with actual concentrations (±SE) of 0.09±0.006 (control), 2.9±0.4 (low) and 6.8±0.6 (high) µg Se/g dry weight. Diets were analyzed using atomic absorption spectroscopy, in triplicate. To prepare the diets, the appropriate amount of selenomethionine was dissolved in approximately 100ml of distilled water and added to the fish food. The mixture was stirred for approximately 10 minutes,

and additional aliquots of 200 ml of distilled water were added until the diet achieved a uniform consistency. The mixture was frozen at -20° C for 48 h and then lyophilized to extract the water. Dried diets were stored at -20° C for the duration of the experiment. All fish were fed once daily, ad libitum. In part A, all fish received a 36-day exposure to one of the three diets. In part B, half of the fish received a 35-day exposure and half of the fish received a 70-day exposure to one of the three diets. After their respective exposure periods, half of the 35-day and 70-day fish began a 42-day recovery period where all treatment groups received the control diet once per day.

Kinematic Analysis

Burst escape chambers were comprised of a 66 by 66 cm corrugated plastic tank with a clear acrylic base, with a 63 cm diameter clear circular plastic insert to keep the fish out of the corners of the arena. The arena was illuminated with strips of LED lighting on the outside of the plastic walls to provide even and diffuse lighting. Water in the chamber was kept at a depth of approximately 9 cm to minimize movement on the vertical axis, and at a temperature of 20° C, equivalent to the water the fish were held in. Above the centre of the arena, a tapered weight (70 g, 3 cm diameter, 12 cm length) was suspended inside an 85 cm tube with an 8 cm opening. The weight was controlled by a button-actuated electromagnet to remotely allow its release, see figure 2.1. The weight was tethered with fishing line so that at full release, the tip of the weight just broke the surface of the water. A mirror at a 45° angle placed underneath the clear-bottomed chamber allowed for the fish response to be recorded with Casio Exilim EX-ZR100v high-speed camera at 480 fps.

At the end of their respective exposure periods, fish were individually placed into the burst escape chamber and allowed to acclimate for at least seven minutes. After the acclimation period, the weight was dropped when three conditions were satisfied: 1) the fish was within 20 cm of the centre of the arena, 2) the fish was oriented with its head towards the tube opening with a 45° angle buffer on each side and 3) the fish was not travelling forward, and its position was being held by movement of the pectoral fins. The fish must elicit a C-start response that is obviously from the weight for the trial to be valid. If an early response from an unforeseen external stimulus occurred, the test would be rerun after an additional acclimation period and once the fish was calm enough to proceed.

After each trial, fish were euthanized using an overdose of MS-222 followed by a blow to the head, in accordance with our animal use protocol. Fork lengths were collected post-mortem. The carcasses were collected and stored at -20° C for selenium analysis.

Video Analysis

Image processing occurred using ImageJ software (version 1.50i). Video files were converted into stacks of JPEG images. Images were then converted into 32-bit black and white and then the "Find Edges" function was used to make the outline of the fish more obvious to the image processor. The scale was established by measuring the opening of the tube across four axes and averaging the value. The tip of the fish's head was tracked over the first 15 frames (31.25 ms), approximately the time required to complete the first two phases of a C-start behaviour (Domenici and Blake 1997). Maximum velocity, maximum acceleration, distance travelled, latency to respond, escape angle and body bend angle were calculated from the image files. The software provided distance travelled per frame and velocity in each frame. Acceleration was determined by using the change in velocities between two frames. Latency to respond was determined by taking the difference between the frame when the weight first broke the surface of the water and the frame when the fish first movement was observed. Each frame was approximately 2 ms. Escape angle was the angle between the final escape trajectory and the stimulus. Body bend was measured as the angle between the head and the tail, through the midsection, in the frame where the magnitude was greatest. All image processing was carried out with the processor being blind to the treatment groups.

Selenium Analysis

Samples of the experimental diets and the whole fish samples were thawed at room temperature, weighed and transferred to conical bottom polyethylene tubes. Analysis of the whole fish was chosen as the escape behaviours of interest are a cumulative response of a number of body systems including sensory, motor and neural systems. Samples were digested with nitric acid (8 N, in an approximately 1:5 tissue weight to acid volume ratio) for 48 h at 60° C, following the methodology of Misra et al. (2012). Samples were diluted with 0.2% nitric acid prior to total selenium measurement by a graphite furnace atomic absorption spectroscopy (AAnalyst 800, Perkin Elmer, USA). The quality control and quality assurance of the analytical method were maintained by using a certified selenium standard, the standard addition and

recovery procedure, and a certified reference material (DOLT-4; National Research Council of Canada) (87% to 110% recovery rate).

Statistical Analysis

In part A, a one-way MANOVA was used to assess if the selenomethionine-spiked diets (control, low and high diets) had a significant effect over a control diet on the response variables of the fast-start response (maximum velocity, maximum acceleration, total distance travelled, body bend, and escape angle). The Pillai's Trace test score was used to determine the significance of the variances, as it is the most conservative and robust to deviations from the assumptions of a MANOVA. Once a significant effect was detected, a series of one-way ANOVAs were used to assess what variable displayed the significant difference. Finally, post-hoc Tukey HSD tests were used to determine what treatment groups were different within the ANOVA. The change in threat perception, as measured by the latency to respond to the stimulus, was assessed using a one-way ANOVA. All response variables displayed homogenous variance and all displayed normality, except for latency to respond. ANOVAs are robust to violations of normality, so I proceeded as normal with all response variables.

In part B, a two-way ANOVA was used to assess if exposure period (35 and 70 days) and selenomethionine -spiked diets (control, low and high selenomethionine diets) had a significant effect on the whole-body selenium concentrations of the Fathead Minnows. A two-way MANOVA was used to assess if exposure period (35 and 70 days) and selenomethionine-spiked diets (control, low and high selenium diets) had a significant effect on the response variables of fast-start response (maximum velocity, maximum acceleration, total distance travelled, body bend, and escape angle). The Pillai's Trace test score was used to determine the significance of the variances. To assess the change in threat perception, as measured by the latency to respond to the stimulus, a two-way ANOVA was used to compare our exposure periods and diet concentrations. All response variables displayed homogenous variance and all displayed normality, except for max velocity, max acceleration, escape angle, and latency to respond. ANOVAs are robust to violations of normality, so I proceeded as normal with the statistical analysis for each variable. To assess the effect of the recovery period on the response variables, I utilized a series of multiple regression analyses. My response variables were the five kinematic variables mentioned above and the latency to respond to the stimulus. The explicative variables

were my three diet treatments and the two exposure length treatments. I included all variables, regardless of significant change at the end of the exposure period, to ensure that I did not miss a latent change to any of the variables. All statistical tests were run using R software ver. 3.3.1 using RStudio ver. 1.0.136, and figures were generated using the ggplot2 package (Wickham 2009).

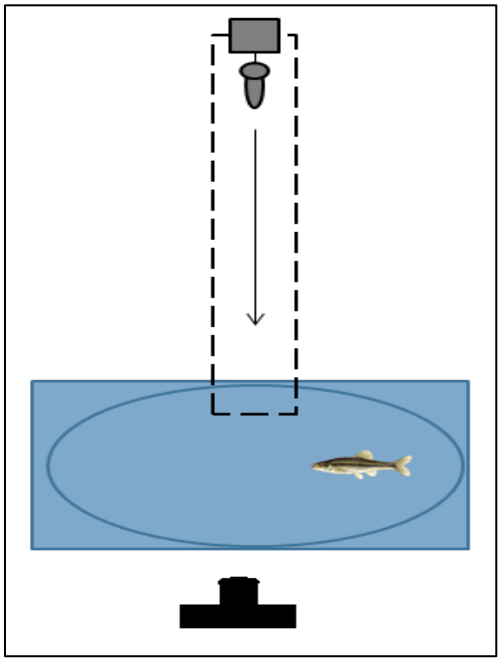

Figure 2.1 A visual representation of the experimental apparatus used to test the kinematic response to a mechanosensory stimulus. The stimulus falls towards the fish from above the tank. The trial is recorded by highspeed camera, from underneath.

2.3 Results

Part A

I observed a significant effect of diet on the kinematic response of the Fathead Minnow (Pillai's Trace = 0.42, $F_{2,42}$ = 2.1, p = 0.03). Subsequent univariate ANOVAs revealed that body bend angle was the only response variable that was significantly different among treatment groups ($F_{2, 42}$ = 5.4 p=0.008, figure 2.1). The low treatment fish had a significantly higher angle of body bend, meaning that they did not display as tight of a "C" shape during the escape as the high treatment group (p=0.006), but not the control group (p= 0.29). There was a 17% decrease in body bend angle between the low and high doses. The remaining variables did not display a

significant difference ($p \geq 0.09$), see table 2.1. There was no significant effect of diet on the latency to respond to the stimulus ($F_{2, 42} = 0.14$, $p = 0.87$).

Part B

There was a significant effect of diet ($F_{2, 32} = 40.8$, $p < 0.001$), but not exposure period ($F_{1, 32} = 0.4$ $p = 0.49$), on the selenium concentrations of the whole-body (wet weight) samples analyzed, see figure 2.2. There was no significant interaction between diet or exposure period on the concentrations of selenium within the fish ($F_{2, 32} = 2.7$, $p = 0.08$). I did not observe any significant effects of either diet (Pillai's Trace = 0.05, $F_{2,144} = 0.55$, $p = 0.9$) or exposure period (Pillai's Trace = 0.03, $F_{1,144} = 0.67$, $p = 0.7$) on the kinematic response of the Fathead Minnow. There was no interaction between the factors of the MANOVA (Pillai's Trace = 0.05, $F_{2,144} = 0.56$, $p = 0.9$). There were no observed positive or negative trends between our treatment groups and the recovery period ($p \geq 0.17$), see table 2.2. There was no significant effect of diet ($F_{2, 144} = 0.35$, $p = 0.70$) or exposure period ($F_{2, 42} = 0.10$, $p = 0.76$) on the latency to respond to the stimulus. There was no positive or negative trend between on our treatment groups and the recovery period ($F_{2, 190} = 0.96$, $p = 0.43$, Adj. $R^2 < -0.01$).

Table 2.1 A summary of the results from the univariate ANOVAs of the kinematic variables from the fast-start response of the Fathead Minnow, in chapter 2 - part A.

Variable	F-Statistic	Degrees of Freedom	p-value
Maximum Velocity	2.2	2, 42	0.13
Maximum Acceleration	0.28	2, 42	0.76
Total Distance	2.2	2, 42	0.12
Escape Angle	1.9	2, 42	0.16
Body Bend Angle	5.3	2, 42	0.008

Table 2.2 A summary of the statistical outputs from the multiple linear regressions on the kinematic variables of the Fathead Minnow's fast-start response.

Variable	F-Statistic	Degrees of Freedom	p-value	Adjusted R^2
Maximum Velocity	0.72	4, 190	0.58	-0.006
Maximum Acceleration	0.87	4, 190	0.48	-0.003
Total Distance	1.6	4, 190	0.17	0.01
Escape Angle	0.34	4, 190	0.85	-0.01
Body Bend Angle	1.4	4, 190	0.23	0.008

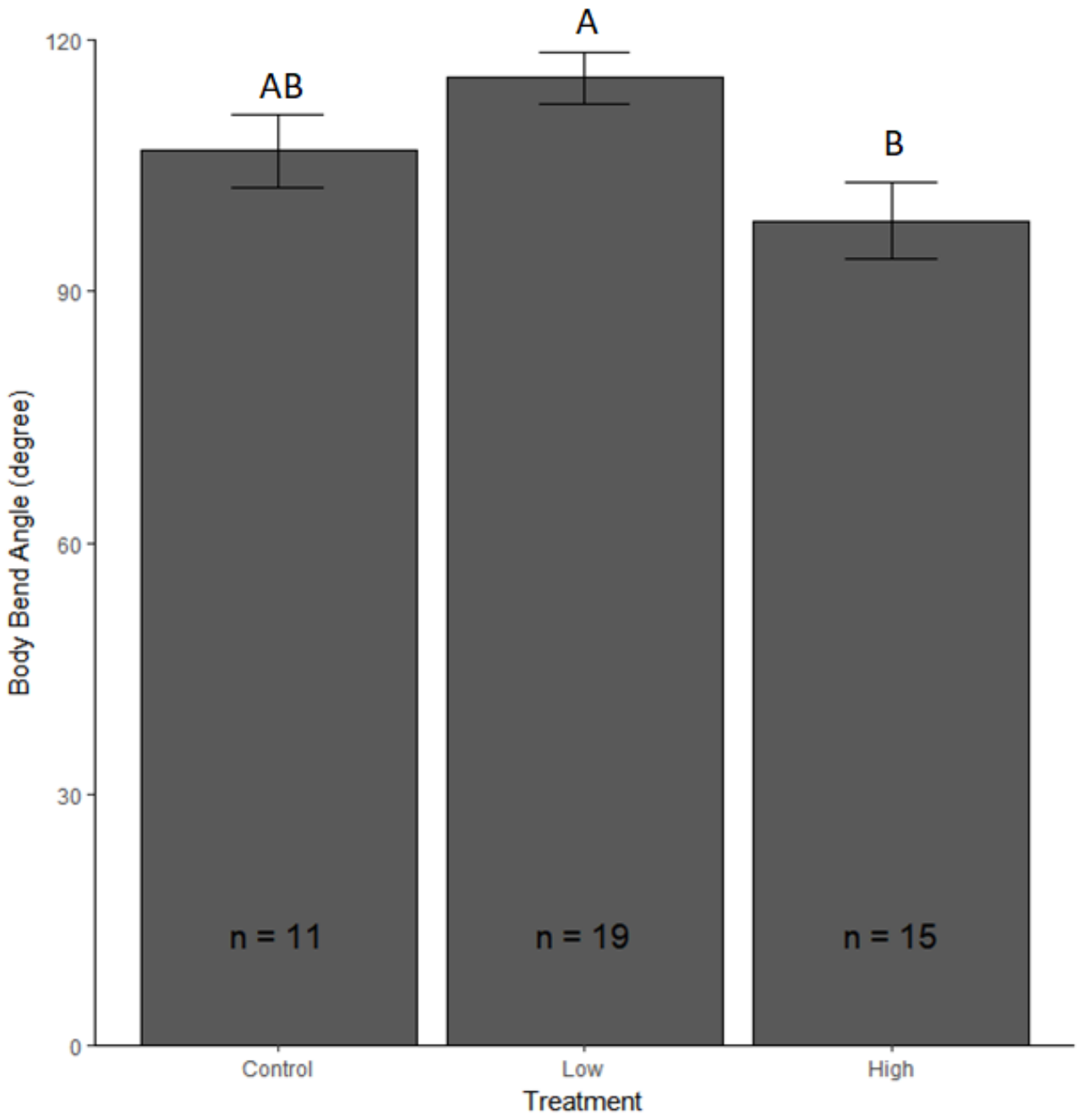

Figure 2.2 The mean (±SE) body bend angle of the Fathead Minnow when exposed to various selenomethionine-spiked diets for a 36-day exposure period. A low value of body bend indicates a strong "C" shape in the first phase of the response, and a high value indicated a weak "C" shape, closer to straight line. Sample size for each treatment group is indicated in black within the bar. Letters denote significant difference between treatments ($p<0.05$).

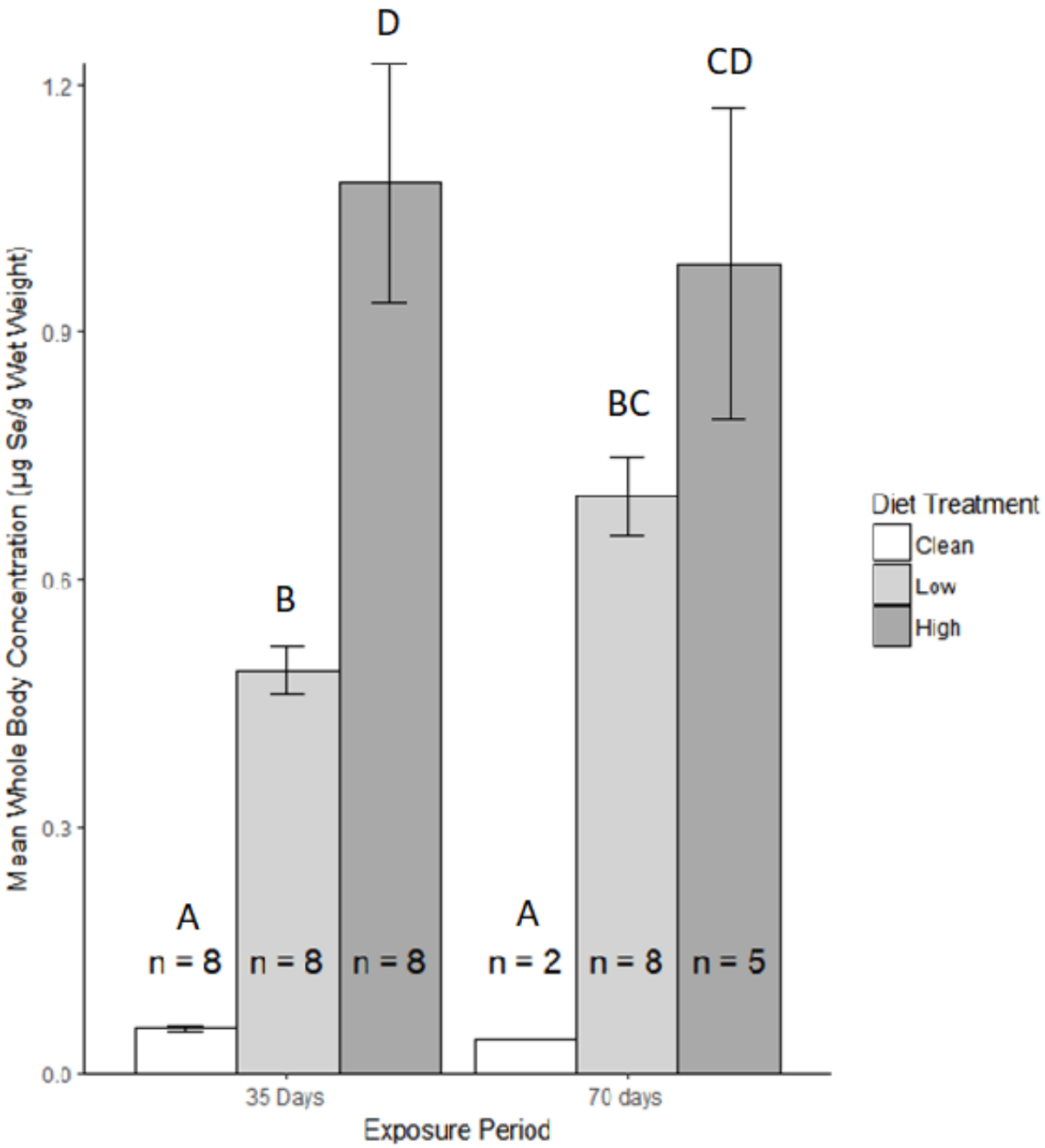

Figure 2.3 The mean (±SE) whole-body concentration of the Fathead Minnow when exposed to various selenomethionine-spiked diets for either a 35- or 70-day exposure period. Selenium concentrations were analyzed using atomic absorption spectrometry analysis. Sample size for each treatment group is indicated in black within the bar. Letters denote significant differences between treatments ($p<0.05$).

2.4 Discussion

In part A, I observed that a selenomethionine-spiked diet led to a decrease in the body bend angle during the characteristic "C" shape of the fast-start response. The high treatment fish had a significantly lower mean bend angle than the low treatment fish, but not the control. In part B, I observed no effect of a selenomethionine-spiked diet on the kinematic response and the latency to respond to a threat on Fathead Minnows at environmentally relevant concentrations. There was no change in any of our observed variables across the recovery period.

The only variable I observed to be significantly affected by the selenomethionine-spiked diet was the magnitude of the body bend during the first phase of the C-start response. I observed a slight hormetic response with this variable, a 7% increase in body bend angle for the low dose relative to the control and a 17% decrease for the high dose relative to the low dose. A hormetic or inverted U-shaped response is where a low dose inhibits and a high dose stimulates the variable relative to a control dose. A high body bend angle would suggest that the fish does not have as strong of a "C" shape during the response. Fish receiving a low dose of selenomethionine were unable to complete a deeper bend, like the high and control dose fish, but did not significantly alter the other variables I observed. This suggests there may be a slight effect on the ability to bend, but is compensated by the other components and has little effect on the whole response. The duration of the turn was not observed in this study, but a large body bend angle with a longer turning period would likely have the same overall effect as a small body bend angle over a short period. A focused investigation into the timing and directional components in the fast-start response could lead to interesting insights on how stressors force fish to compensate for shortcomings in one area.

Dietary selenomethionine has an impact on critical swimming performance in both Fathead Minnows and Zebrafish (*Danio rerio*) (Thomas and Janz 2011, McPhee and Janz 2014). Critical swimming performance is a common measure of aerobic swimming capacity. My study focused on the burst swimming performance, which is typically fuelled by anaerobic respiration (Jayne and Lauder 1994). McPhee and Janz (2014) postulated that the decrease in swim performance was linked to energy metabolism and muscle function. Aerobic and anaerobic respiration use different molecules for fuel, primarily triglycerides and glycogen (Jayne and Lauder 1993). Selenium may be impacting the way fish catabolize triglycerides but have no

effect on glycogen. If selenium is impacting the mechanisms that fish use to burn the fuel required for movement, the longer the duration of the movement, the more obvious the impact may be. The increased duration of sustained swimming may lead to a depletion of energy stores and production of metabolic waste products that slow the motor function. In burst swimming of selenomethionine exposed fish, fuel may be consumed or waste produced at a similarly elevated rates, compared to control fish, but the short duration prevents complete depletion of fuel or saturation of metabolic wastes to such a point where they hinder the performance. If oxidative damage has altered the pathways for the breakdown of waste products, a more rapid fatigue may be experienced in prolonged swimming behaviours.

This dissimiliarity between anaerobic and aerobic performance has been previously noted in the literature. Rajottee and Couture (2002) observed that in wild-caught fish from metal contaminated lakes near Sudbury, Ontario, the performance in a steady state swimming test decreased as contamination level increased, but burst swimming performance did not. The study utilized critical swimming speed and distance travelled during an electrically stimulated fast-start to compare the performance of these two types of swimming (Rajotte and Couture 2002). A mixture of metal contaminants were found in these lakes, and therefore the ultimate cause of toxicity in the fish is likely different than in fish solely exposed to selenium. However, it does suggest that the assumption that when one toxicant affects a swimming behaviour, it will equally affect another, is incorrect.

In this study, I present wet weight for all selenium concentrations. The utilization of dry weight measurements is another convention. The U.S. EPA presents a standard moisture content for Fathead Minnows at 76.6%, meaning that a dried sample will have 3.3 times more selenium in it than a wet sample of the same mass (United States Environmental Protection Agency 2016). See table 2.3 for a conversion of our wet weight values to dry weight, to allow for comparison to other studies in the literature. A standard conversion ratio between wet and dry weights can be used, but an analysis to determine the specific ratio of the experimental fish is preferred, as the standard values can be skewed due to changes in life history or body tissue composition (Cresson et al. 2017). Our whole-body concentrations are lower than other studies currently published, which may be due to a number of factors, including type of feed, life history stage, or species (Hamilton et al. 1990, Thomas and Janz 2011, McPhee and Janz 2014). However, our high

treatment fish had a whole-body concentration close to the tissue-based guideline recommendation and currently British Columbian regulation limit of 4 µg/g dw (Hamilton 2002). The goal of this study was to assess the impacts of environmentally relevant concentrations of selenium on the escape behaviour of fish. By using fish with body burdens less than the regulatory limits, I replicated the conditions fish experience in nature to assess if the protection provided by regulations is adequate.

In summary, the findings of this work indicate that there was no consistent significant effect of selenomethionine on the fast-start response of the Fathead Minnow for the exposure period and concentrations of this study. There is evidence in the literature that selenium can have significant impacts on the steady state swimming behaviour of Fathead Minnows and other freshwater fish (Thomas and Janz 2011, McPhee and Janz 2014). The hypothesized mechanism for decreased swim performance was the metabolism of energy in selenium-exposed fish. The differences in energy metabolism in aerobic swimming and anaerobic burst movements may contribute to the differences in our findings. Dissimilarities between changes to aerobic and anaerobic swimming in the face of contaminants have been noted in past (Rajotte and Couture 2002). A follow-up to this experiment could test both the aerobic and anaerobic swimming capacity of freshwater fish to determine how exposure to selenomethionine may specifically impact different motor functions.

Table 2.3 Conversion of selenium concentrations of whole-body samples from wet weight to dry weight. 76.6% moisture content used for conversion taken from the US Environmental Protection Agency report (2012). Exposure periods are reported in days. Selenium concentrations are mean values ± S.E.M. and are presented as µg Se/g.

Diet	Exposure period	[Se] Wet Weight	[Se] Dry Weight
Control	35	0.05±0.003	0.2±0.01
	70	0.04±0.00007	0.1±0.0002
Low	35	0.5±0.02	1.6±0.1
	70	0.7±0.05	2.3±0.2
High	35	1.1±0.1	3.6±0.5
	70	1.0±0.2	3.2±0.6

Chapter 3: The effect of dietary selenomethionine on the ability of Fathead Minnows to respond to a looming threat

3.1 Introduction

As stated in the previous chapters, selenium is an important anthropogenic stressor to aquatic systems. Teleost fish rely on their escape response to increase their likelihood of survival during a predatory strike. The escape response is triggered by a neural response from the Mauthner cell system (Eaton et al. 1977). This response can arise from a number of different stimuli, such as a weight drop, a sound, or a visual stimulus (Domenici and Blake 1993, Allan et al. 2013, Simpson et al. 2015a).

Fast-start response initiation is a complex process in fish. It is well-known that fish can respond to a visual looming stimulus as they would respond to electrical or vibration stimulus. A rapidly approaching dark circle on a light background was shown to be the most effective stimulus at eliciting an escape response in larval fish (Temizer et al. 2015). The reaction is triggered by a signal through the tectal retinal ganglion cells' axons that travels into the Mauthner cell system (Temizer et al. 2015). Lesions on these cells completely removed the fish's ability to respond to a visual stimulus, indicating they are an essential component of the neural pathway responsible for escape responses (Temizer et al. 2015). A fish's ability to escape predators is extremely important, and a change in ability to process visual stimuli could have serious impacts.

The use of a looming visual stimulus to study the escape response of fish is a standard methodology to test predator avoidance ability (Batty 1989, Fuiman and Cowan 2003, Simpson et al. 2015a). As mentioned above, a black circle increasing in size on a white background will elicit a fast-start response from a calm fish. Isolating the visual component of the stimulus required for a fast-start response may not seem to be ecologically relevant, as a predatory strike consists of more than just a visual component. However, by examining specific components of the fast-start response, I can achieve a better understanding of how selenium impacts this behaviour. The eyes of fish have been of special interest in the study of selenium toxicity. Cataracts and exophthalmos are symptoms of selenium toxicity in wild populations that can stunt a fish's ability to detect visual changes in the environment (Lemly 2002) and selenium has been shown to accumulate preferentially in developing eye tissue (Choudhury et al. 2015). By

focusing on the visual portion of the escape response, I can determine if selenium toxicity is having a significant effect on the eyes and their influence on the escape response as a whole. It has been shown that an increase in the latency to respond can detrimentally affect fish survival in predation events (Nair et al. 2017).

In this experiment, I investigated the effects of dietary selenomethionine on the ability of Fathead Minnows to respond to a looming visual stimulus. As in chapter 2, I exposed minnows to selenomethionine-spiked diets for various sub-chronic exposure periods. I proceeded to test the fish in a visual looming stimulus chamber to assess the latency to respond to a visual threat with a C-start response. I also investigated the ability of the fish to recover from the selenium exposure when a clean diet is reintroduced. I hypothesized there will be a significant effect of selenomethionine on the ability to respond to a visual stimulus. I predicted that I will observe an increased latency to respond to the stimulus in the selenomethionine-exposed fish. It has been shown that visual impairment is a symptom of selenium toxicity in freshwater fish, and that selenium preferentially accumulates in the eye lens of developing fish and in the brain of adult fish, suggesting that selenium can impede visual sensing (Lemly 2002, Misra et al. 2012, Choudhury et al. 2015). I also hypothesized that the fish will display a significant change in the ability to respond to a visual stimulus over the recovery period. My prediction was that as the selenium is depurated by the Fathead Minnow (Bertram and Brooks 1986), the fish will recover from the stressor and return to a normal latency to respond over the course of the recovery period.

3.2 Methods

Study Species Collection and Care

All Fathead Minnows used in this experiment were also used in chapter 2 - part B. Whole-body concentrations for each treatment group were presented in the last chapter. Fish were first run through the fast-start procedure, and then through the looming threat procedure within the same day. The experiment ran concurrently with chapter 2 - part B, May 15th to September 13th, 2017. Fish were housed prior to the experiment according to the procedures outlined in the previous chapter.

Diet Preparation and Exposure

Diets for this experiment were the same as in chapter 2 - part B, with actual concentrations of 0.09±0.006 (control), 2.9±0.4 and 6.8±0.6 µg Se/g dry weight. The diets were prepared as described in chapter 2 – part B. Each fish received one of the three diets for either 35 or 70 days, as in chapter 2 - part B. The recovery period and procedures were the same as in chapter 2 - part B.

Looming Stimulus Analysis

The visual looming stimulus apparatus consisted of a 6.8 L glass aquarium on a benchtop with a metal frame above. The aquarium was divided in half with white corrugated plastic to keep the fish in the half of the aquarium closest to the stimulus. The side of the aquarium furthest from the observer was wrapped in opaque, black plastic, and the near side was wrapped with dark grey window tinting (5% visual light transmission). The end facing towards the stimulus was clear so the fish could see the approaching disc. The tank was illuminated from above and the side. Attached to the metal frame above the tank was a 75 cm wooden dowel with a 10 cm diameter white disc with a 4.5 cm diameter black circle in the centre. This disc was pulled back 42 cm and attached to an electromagnet. The button-actuated electromagnet allowed the disc to swing freely when released. A tether connected the disc to the magnet to prevent the disc from striking the tank, see figure 3.1. This produced a visual stimulus of a black circle increasing in size to the fish, which elicited a fast-start response, similar to Fuiman and Cowan (2003) and Simpson et al. (2015a). Each fish was tested singly, and allowed approximately 10 minutes of acclimation in the tank prior to testing. The magnet was released when the fish appeared to be calm, holding position, or slowly moving with its pectoral fins, oriented with its head towards the stimulus, and closer to the open side of the tank than the divider in the centre of the tank. If the fish did not clearly respond to the stimulus, they would be rerun, up to two additional times. Only the successful response would be considered for analysis. All trials were recorded from above at 480 fps, with a high-speed (Casio Exilim EX-ZR100) camera positioned so that both the experimental chamber and the disc were always visible.

Video Analysis

Image processing occurred with ImageJ software (version 1.50i). Video files were converted into stacks of JPEG images. The latency to respond to the visual stimulus was measured as the difference in frames numbers between the first movement of the disc and the initiation of the fast-start response in the minnow. Each frame is approximately 2 ms. The observer was blind to all treatment groups during this processing step.

Statistical Analysis.

To analyze the effect of my experimental diets and the exposure period, I used a two-way ANOVA. My data did not display normality, but it did display equal variance. I proceeded with the ANOVA as normal, as they are robust to deviations from normality. To assess the effect of the recovery period on the ability of the fish to respond to a visual threat, I utilized a multiple regression analysis. My response variables were the five kinematic variables and the latency to respond to the stimulus. The explicative variables were my three diet treatments and the two exposure length treatments. Statistical tests were run using R software ver. 3.3.1 using RStudio ver. 1.0.136, and figures were generated using ggplot2 (Wickham 2009).

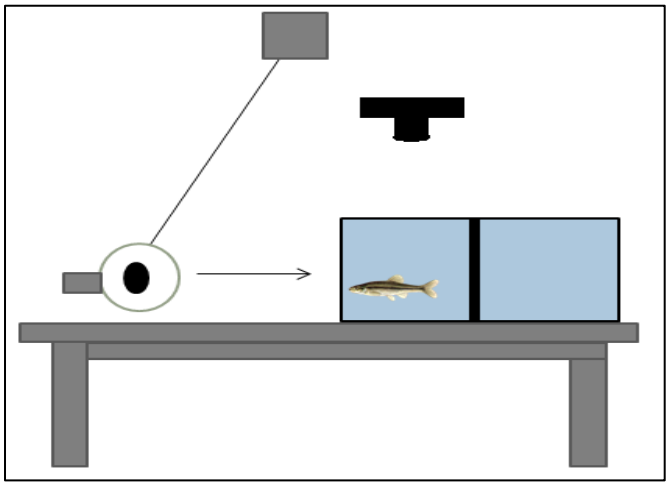

Figure 3.1 A visual representation of the experimental apparatus used to test the latency to respond to a visual looing stimulus. The stimulus swings towards the tank and the trial is recorded from above by high speed camera.

3.3 Results

I did not observe a significant effect of diet ($F_{2, 151} = 0.07$, $p= 0.9$) or exposure period ($F_{2, 151} = 0.17$, $p= 0.7$) on the fish's latency to respond to the visual threat, see figure 3.1. Moreover,

there was no significant interaction between diet and exposure period ($F_{2, 151} = 0.03$, p= 0.9). There was no effect of recovery on the fish's ability to respond to the visual threat ($F_{3, 153} = 0.21$, p= 0.9, Adj. $R^2 = -0.02$) see figure 3.2.

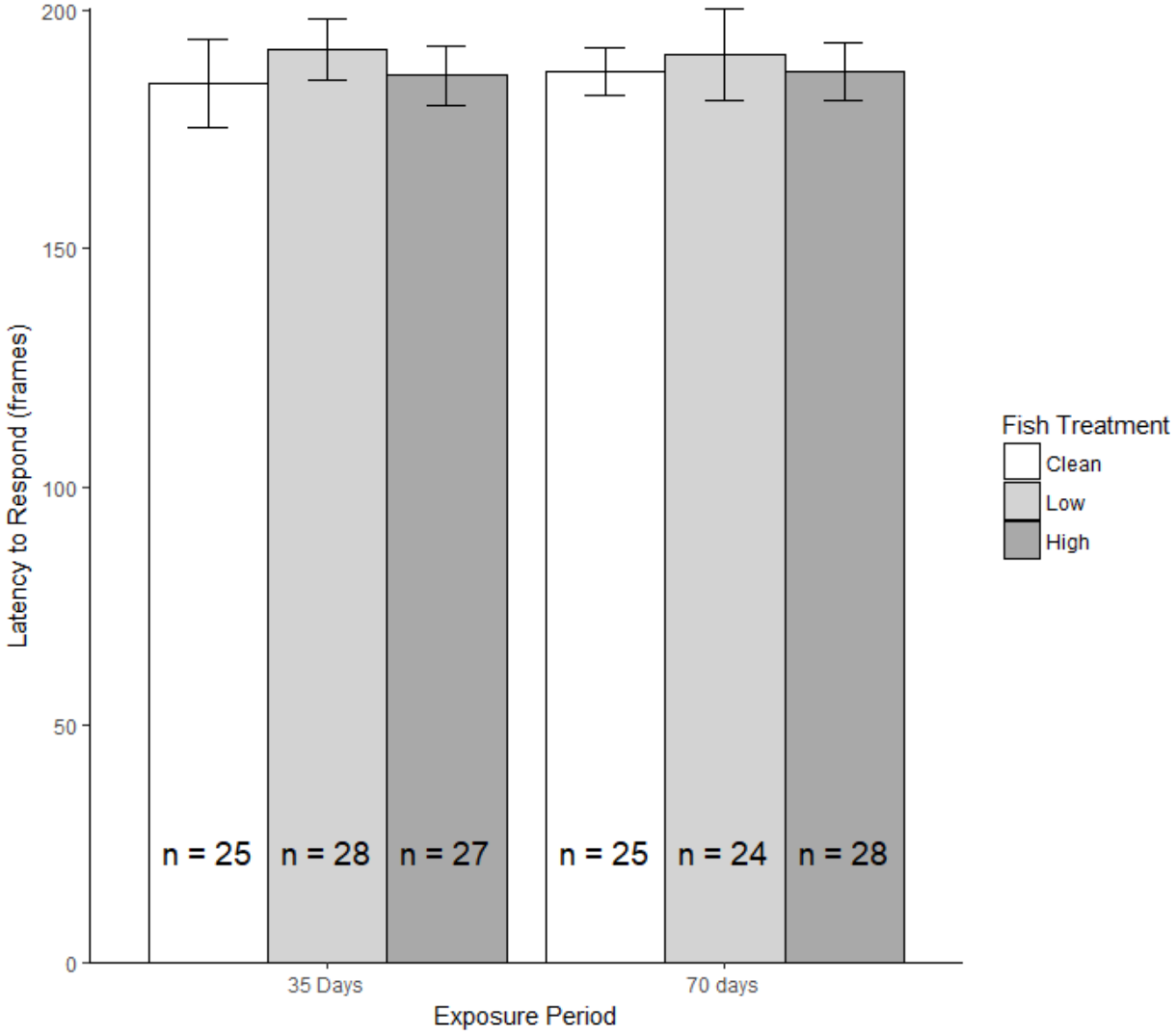

Figure 3.2 The mean (±SE) latency to respond to a visual looming stimulus for Fathead Minnows when exposed to various selenomethionine-spiked diets for either a 35- or 70-day exposure period. Latency to respond is the period between the first detection of movement of the stimulus and the first movement of the fish. Sample size for each treatment group is indicated in black within the bar.

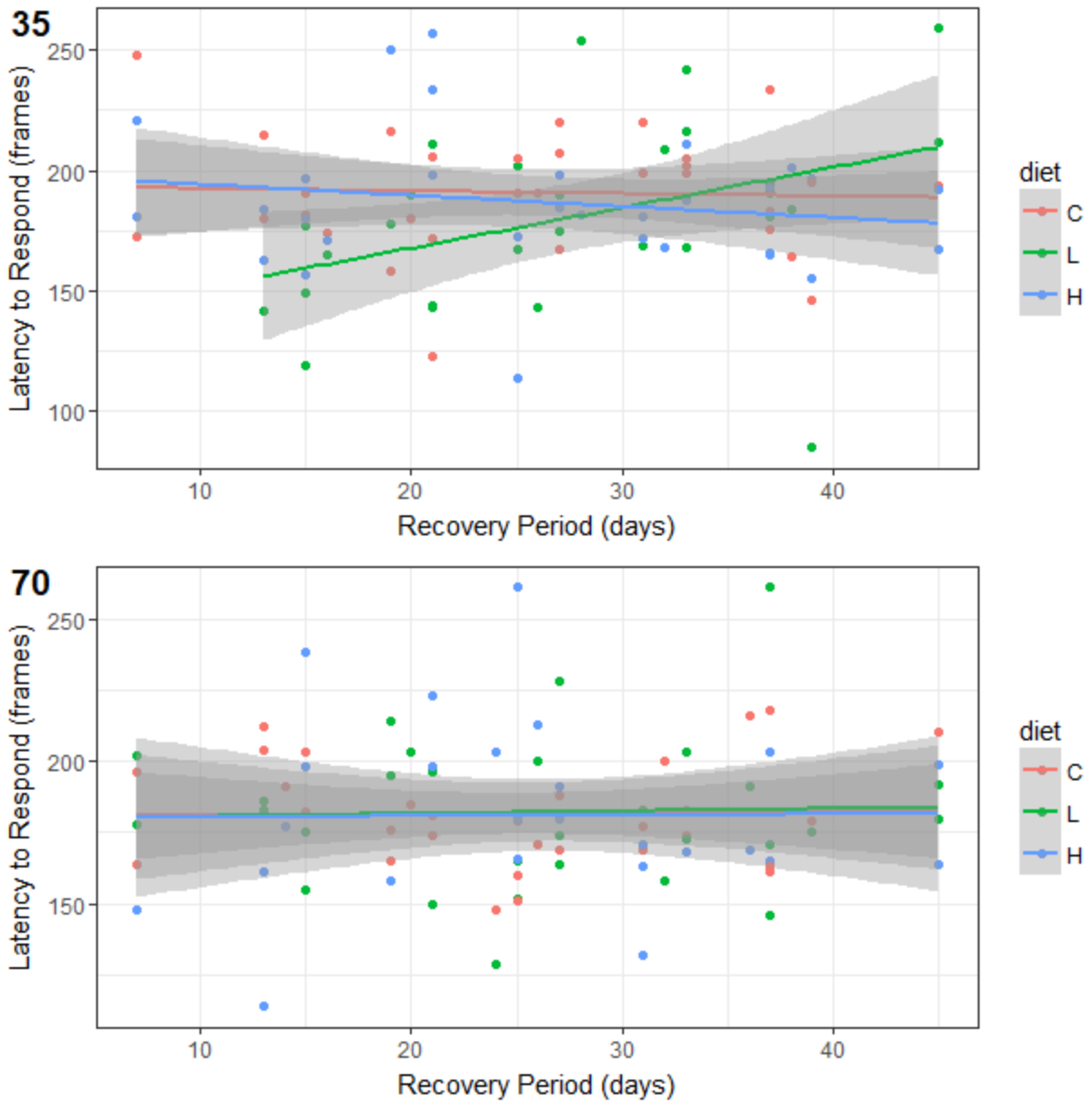

Figure 3.3 The scatter plots of Fathead Minnow latency to respond to a visual looming stimulus when fed one of three selenomethionine-spiked diets, followed by up to 46 days of recovery where they received the control diet. The top panel displays fish that received a 35-day exposure to the experimental diet and the bottom panel displays fish that received a 70-day exposure. The colored lines represent the line of best fit for each treatment, with the standard error in grey. Latency to respond is the period between the first detection of movement of the stimulus and the first movement of the fish.

3.4 Discussion

There was no significant effect of the selenomethionine-spiked diet on the response to a visual looming stimulus in the adult Fathead Minnow. I did not observe a change in the response over the course of the recovery period. The whole-body concentrations of the fish are at and below the levels presented in Canadian tissue-specific guidelines, see chapter 2 section 3 for details. At the current regulation levels, I do not predict an effect of selenium on the visual processing of a looming threat in adult Fathead Minnows. However, this does not mean that the levels of selenium exposure used in this experiment will not affect the survival or biological functioning of freshwater fish.

In the literature, there are studies on other environmental stressors that affect the ability of fish to perceive visual threats. Noise, metals, and persistent organic pollutants have all been shown to decrease the visual acuity of fish, using similar experimental procedures as my study (Faulk et al. 1999, Rice et al. 2011, Simpson et al. 2015a). These studies state that decreased startle responses may have an important impact on the survival of the fish. From chapter 2, I am aware there are differences in the observed effect of selenomethionine on steady state swimming and burst swimming behaviours. Conflicting results have also been observed between routine swimming and visual startle responses. The herbicide atrazine leads exposed fish to swim faster, travel further distances, and along more convoluted paths, but does not have an effect on the visual startle response (Alvarez and Fuiman 2005). Alvarez and Fuiman (2005) stated there will be an effect on the fish's survival due to changes in routine behaviour, through increased predator encounters and energy requirements, despite escape behaviour not changing. While my study did not demonstrate a change in the escape behaviours at environmentally relevant concentrations, other work has shown that sustained swimming is compromised (McPhee and Janz 2014). Therefore, I can predict that overall survival of affected fish will be decreased when selenium contamination occurs in a water body. The changes to specific swimming behaviours are subtle, and cannot be generalized to all aspects of a fish's performance. I provide further evidence that swimming behaviours cannot be generalized with this study.

Most of the studies on fish visual startle behaviour expose fish to stressors either via parental exposure or during development (Faulk et al. 1999, Rice et al. 2011, Simpson et al. 2015a), while my study utilized sub-chronic exposures to adult fish. When considering chemical

toxicants, the differences between my work and the literature may suggest that fish in developmental periods, either in ovo or post-hatch, are more susceptible to deleterious effects on their vision than adults. There are a wide variety of contaminants that are harmful during development and less so during adulthood, and selenium is likely included on that list (McKim 1977, Lemly 2002, Liney et al. 2005, Zhao et al. 2014). It is well-known that environmental selenium exposure has significant impacts to the behaviour, physiology and the contaminant loads of developing fish (Lemly 1997, Muscatello et al. 2008). Adult fish are less often focused on in the ecotoxicology literature, with most examples focusing on field studies and in situ caging experiments (Lemly 2002, Phibbs et al. 2011). Adult fish display significant mortality in experimental streams under chronic exposure to elevated selenium levels, but their progeny display similar mortality at lower concentrations and exposure lengths (Hermanutz et al. 1992). My diet concentrations and exposure periods were similar to those in McPhee and Janz (2014), but juvenile fish were used. My exposures on adult fish resulted in whole-body concentrations that were lower. My work suggests the adult Fathead Minnow has different tolerances to environmentally relevant exposures of selenium compared to developing fish.

The eyes of fish do not develop like those of mammals. They are continually growing throughout the animal's life (Hitchcock et al. 2004). The mass of the human eye increases twenty-fold over the course of its life, while the eye of the Zebrafish increases one million-fold (Hitchcock et al. 2004). This continued development suggests that adults should be susceptible to damage, as a larval or embryonic life stage would. It has been noted that Zebrafish visual acuity can be compromised in both adult and larval fish exposed to selenium, albeit in different ways (Raine et al. 2016). The adults displayed a decrease in only escape response initiation, while larval fish decreased in a broader range of tests for phototaxis, optomotor and optokinetic responses. I did not observe an effect of selenium, at the exposure conditions of the study, on the latency to respond in adult Fathead Minnows. The lack of an observed impact during adult stages may suggest that damage done during the preliminary development is more serious. The rapidly differentiating embryonic cells may not have the capacity to repair selenium substitutions to disulfide bonds or have increased susceptibility to oxidative damage. The development of the structure of the eye during embryonic development may be at greater risk of damage or mutation that leads to loss of function. The continued development of the adult eye tissue is described as circumferential additions, slowing as the fish ages (Johns 1982). Fish retinal neurons have the

capacity to regenerate when damage or death of the cell occurs (Hitchcock et al. 2004)w. As the development of cells slows, damage to new cells may not leave a lasting impact as the capacity for repair is not overwhelmed. To tease apart the relationship between life stage and the prevalence of damage to eye tissue, further work is required. An investigation into how selenium damages the eye tissues during larval development versus during adulthood could explain the differences observed in the visual acuity of adult and larval fish.

In conclusion, I observed no significant effect on the response to a visual stimulus in adult Fathead Minnows, at environmentally relevant concentrations of selenium. However, there are likely still impacts to the survival of fish due to the other impacts of selenium on behaviour, but that is beyond the scope of this study. Maximum whole-body concentrations of selenium in the fish of this study approach the Canadian tissue-specific regulatory levels (DeForest et al. 1999, Hamilton 2002), making for an accurate study of how fish in real-world conditions are responding to legally allowed levels of pollution. Moving forward, a study focused on the change of predator-prey dynamics could demonstrate how the change in swimming behaviours alters community interactions in a real-world setting.

Chapter 4: General Discussion

4.1 The impact of selenium on escape behaviour

My study did not demonstrate a consistent significant effect of a selenomethionine-spiked diet on the escape behaviour of the Fathead Minnow. I observed a single significant impact on the magnitude of the body bend in the first of my experiments, but when a more comprehensive follow-up experiment was carried out, the results could not be reproduced with slightly lower concentrations. There was no evidence that my exposures hindered the fish's ability to perceive a visual threat. Overall, my results did not support my hypothesis that there would be a significant effect of selenium on the initiation or the performance of escape behaviour at the concentrations used.

When one behaviour is affected by a toxicant, there is no guarantee that other, albeit similar, behaviours will be affected in the same way (Rajotte and Couture 2002, Alvarez and Fuiman 2005). Selenium depresses steady state swimming capacity and performance in multiple species of freshwater fish (Thomas and Janz 2011, McPhee and Janz 2014). However, I did not observe an effect on escape response behaviours at the exposure levels used in my thesis. The effect of selenomethionine on visual acuity cannot be generalized. My study failed to demonstrate a significant effect on the ability of minnows to perceive a looming stimulus. However, Raine et al. (2016) have shown that similar durations and concentrations of exposure to selenomethionine altered the visual system, but to different extents in adult and F1 generation Zebrafish. While there were different methodologies used, an approaching disc versus a band on the inside of a rotating drum, in the two studies, the underlying mechanism of the response is similar.

My study adds to the existing literature on the impacts of selenium pollution on freshwater fish. It specifically complements the literature on sustained swimming performance to provide a complete perspective on the effect of selenium on swimming (Thomas and Janz 2011, McPhee and Janz 2014). Using adult fish helps to fill in a knowledge gap, as most of the literature focuses on individuals in developmental life stages. My work suggests that adults are more tolerant of selenium toxicity than larval fish, likely because selenium is not causing oxidative damage during sensitive periods when the sensory and motor systems are developing. A follow-up study comparing the impacts of my exposures on a variety of life history stages

would provide further support to this hypothesis. Using environmentally relevant concentrations allowed me to assess how selenium at real-world concentrations affects fish swimming behaviour. Swimming performance was affected by selenium concentrations slightly higher than the ones used in my thesis (Thomas and Janz 2011, McPhee and Janz 2014). By utilizing lower concentrations than other studies, I was able to investigate the lower threshold for selenium toxicity in Fathead Minnows. Understanding at what level selenium contamination has a negligible effect on the behaviour of freshwater fish will allow for better decision-making pertaining to the release of selenium-containing effluent and the management of accidental releases. Finally, the work provides additional evidence that the kinematic analysis of fast-start response is a valid methodology for assessing the impacts of anthropogenic change on fish. This experiment can detect both change and no change to the response variables (Allan et al. 2017). This is important as it supports that the methodology is sensitive to changes in behaviour, but also robust to false positives. The kinematic analysis of fast-start responses is another method for the ecotoxicologist's toolbox to assess the impacts of toxicants on a wide range of behaviours.

4.2 Ecological relevance

Predation is a strong selecting force acting on fish populations, and prey fish are under this pressure their entire lives. It is well-known that the predator-prey dynamic can affect the behaviour and physiology of both prey and predators (Lima 1998, Ferrari et al. 2010), but it is not well understood how various human-induced stressors alter these relationships. For toxicants that bioaccumulate in aquatic systems, forage fish can be the first vertebrates in the exposure pathway. They are critically important to the transfer of contaminants from plants and invertebrates to higher trophic birds, fish and amphibians, which often have significant cultural, economic, or ecological value.

Isolating the individual behavioural components of the predator-prey dynamic is the first step towards understanding how communities of fish respond to change. My work begins to shed light on the burden of toxicity in this relationship, by asking questions such as: is there a differential effect of selenium toxicity on predator or prey? In this thesis, I present evidence that selenium does not have any impact on the escape behaviour of Fathead Minnows when exposed to selenium at the concentrations used. Therefore, if there was an effect of selenium on predator-prey dynamics, the burden of toxicity would likely fall to the predator. Using the literature

currently in the field, I know that sustained swimming can be altered when exposed to selenium (McPhee and Janz 2014) and can make predictions as to which predators would be most affected by contamination. Pursuit predators, such as a Walleye (Hartman 2009), would likely suffer reduced foraging success if their sustained swimming performance and capacity were decreased. An ambush predator, like the Northern Pike (Harvey 2009), may not have a change in foraging success as the short duration burst motion, like the escape response in Fathead Minnows, is the foundation of their strike.

I investigated how the prey's ability to respond to predation threats, therefore a complementary study would look how the striking ability of predators is changed when exposed to selenium. It would focus on the kinematics of the predator strike and the acquisition of targets to determine how selenium is affecting the piscivorous predator. A field or mesocosm study, using a factorial design of selenium exposed and unexposed prey and predators to assess predator success rates and prey survival, would be the culmination of these projects to validate the laboratory observations in a real-world setting.

The depression of swimming performance and capacity has been said to alter survival in fishes (Weis et al. 1999, Walker et al. 2005, Allan et al. 2013). Some studies take this statement a step further and use mathematical models to show how increasing contaminant load impacts quantifiable variables, such as encounter rate, escape success rate, and strike effectiveness (Alvarez and Fuiman 2005, Nair et al. 2017). I believe that the development of models to help quantify predictions will be useful for the advancement of the field. Testing how the components of an escape response, like velocity, acceleration, and distance travelled change as exposure increases will allow for improved understanding of this system. The complex nature of dose-dependent toxicants prevents extrapolation and even interpolation of the results. Toxicants can depress biological functioning in laboratory settings, but without connecting these effects to increases or decreases in survival or predation success, we will not understand how wild communities are affected. Taking a suite of behaviours and determining how they impact survival using models could help to uncover patterns and correlated factors to simplify the process required to characterize contaminants.

Understanding the balance of community dynamics, how populations within a natural system interact with one another, is key to determining how human impacts are likely to unfold

in the future. Predation is one component of the ongoing complex interaction between species in aquatic ecosystems. Without comprehensive study of basic scientific questions, like how do fish respond to predation threats or how does selenium affect vision in fish, society would be unable to make regulatory judgements to protect ecosystems and human health.

4.3 Using behavioural ecotoxicology to study the implications of anthropogenic development

Behavioural responses to toxicants can be highly specific to life stage and species, and therefore we are unable to generalize the results from similar behaviours or populations. The study of individual behaviours is important to ensure that impacts of toxicants are properly assessed. Behavioural changes can often occur at significantly lower exposures than standard mortality tests (Little and Finger 1990, Kane et al. 2005), making them an ideal characteristic to study how regulated levels of contaminants affect aquatic ecosystems. Once contaminants have been regulated, controlled effluent releases usually do not generate exposures that would induce mortality in the population, however they could still impact biological functions of the system. This brings up the concept of physiological and ecological death.

Physiological death can be measured with ones and zeros, whether an exposure will kill a fish or not. LC50 and LD50 values are based on this system. It is a coarse measurement of toxicity, but allows for rapid assessment of novel substances to determine environmental safety. They establish a baseline level of toxicity, and are used to generate regulations and protections. As I described above, behavioural ecotoxicology is highly specific to life history stage, species, etc., making it difficult to be used when regulations need to be quickly set. I believe that behavioural ecotoxicology aims to measure ecological death, which is much more continuous and could be measured with a spectrum. An exposure to a toxicant may not be lethal to an organism but when essential functioning such as foraging, sexual competition or locomotion are stunted, the population can undergo severe changes to fitness or structure. Sublethal concentrations of a contaminant that do not induce mortality in a population, but alter swimming in larval fish to such an extent that predators can consume entire broods in short order, may be allowed through regulations that only utilize LC50 or LD50. The sensitive endpoints of behavioural ecotoxicology may help to protect the functioning of an ecosystem by weighing

ecological and physiological death together to set reasonable guidelines to adequately protect waterbodies.

Environmental Impact Assessment (EIA) and Cumulative Effects Assessment (CEA) are two components of the rigorous environmental assessment process in Canada. EIA focuses on the effects of a specific project and CEA incorporates other developments in the area. Behavioural ecotoxicology is well-suited to meet the specific challenges of EIA and CEA because it can detect the effects of contaminants at low concentrations. The study of trace contaminant mixtures also benefits from behavioural ecotoxicology, where very small concentrations of toxicants can have additive or synergistic effects that may be unobservable in broader responses.

When paired with models that relate the changes in swimming behaviour to predation events, behavioural ecotoxicology can be used to rapidly assess potential community changes in a system. A comprehensive understanding of how specific concentrations of contaminants impact animal behaviours will allow for insight into how the ecology of the system changes. It could provide us with the specific durations or concentrations of exposures that would lead to significant community change. They may allow for controlled effluent releases during specific periods to minimize the biological effects.

Describing the impacts of toxicants on the behaviour of the animals in an aquatic system could be very useful in community consultations. Explaining how pollution alters the routine behaviours of fish could be easier to understand than the changes to specific sensory or metabolic mechanisms that ultimately alter fitness. The concepts and language required to explain how anaerobic capacity and neural function affect the swimming capacity are more complex than those required to explain how an exposed fish with a slower response time might respond to an incoming threat. Ensuring that the work carried out by scientists is accessible to the public is important, especially with federally funded research. We require a knowledge of how an organism is impacted by stressors across all levels of organization, but conveying a message to the public in a scale that they understand and deal with regularly may be more successful.

Regulations are set to limit contaminant releases to protect the environment (Health Canada and Environment Canada 2015). In a perfect world, effluent would contain zero

toxicants, but unfortunately this is not possible. They are constrained by technical and economic limitations, so they are generally not zero. Behavioural ecotoxicology allows us to approach toxicity with realistic considerations. To make practical decisions about contaminants and the natural world, with realism in mind, we must understand how organisms respond to real-world exposures.

4.4 Future directions and conclusions

Escape responses are primarily an anaerobic muscle function (Domenici and Blake 1997), while constant swimming is an aerobic function (Jayne and Lauder 1994). An experiment where anaerobic and aerobic swimming capacity and activity are compared in the same fish would provide a complete perspective on the issues of selenium and swimming. Swimming capacities could be characterized using the methods in this thesis and those in Thomas and Janz (2011) and McPhee and Janz (2014). If possible, carrying out other tests of burst movements, such as foraging strikes or sprint speeds, and sustained swimming movements, such as maximum sustained swimming speed, would allow for a multivariate assessment of swimming performance in contaminant exposed fish.

Understanding the components of the predator-prey interaction is critical for determining community effects. Using a piscivorous predator, or various predators with distinct foraging strategies, for an experiment to assess the impacts of selenium of the success of predation events would provide the complementary data to my work on prey. An impact to the proportion of successful strikes or the energy required in a long pursuit could have detrimental effects on the individual fitness. Having data on both predator and prey would allow for a holistic assessment of contaminant impacts on freshwater systems. A factorial design experiment where both predators and prey are exposed to selenium would provide a controlled environment to test real-world interactions. Laboratory experiments can be used to determine how the predators and prey are affected by selenium, but a comprehensive field study using translocated populations in contaminated systems would still be required to incorporate any unforeseen external influences on the community.

There does not appear to be a consistent effect of dietary selenomethionine on the initiation or kinematic components of the Fathead Minnow's escape response, at the conditions of my exposure. My exposure mimicked real-world conditions and produced whole body

selenium concentrations that approached the conditions of Canadian tissue-based regulation guidelines. While we did not display an effect of selenomethionine, this level of exposure will still likely have an impact on other behaviours and physiological functions. Impacts to survival and how that would affect population and furthermore community composition still needs to be determined. This work provides a base for future work into the ecotoxicological effects of contaminants on swimming behaviour. Despite behaviour such as steady and burst swimming appearing similar when considered topically, the underlying mechanisms are markedly different. The study of individual behaviours must continue until there is a better understanding of the correlations between behaviours.

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